RayBio® Biotin Label-based Rat Antibody Array 1

For the Simultaneous Detection of the Expression Levels of 90 Rat Proteins in Cell Culture Supernates.

User Manual
(Revised Apr 1, 2009)

(Cat#: AAR-BLM-1-2;
AAR-BLM-1-4)

RayBiotech, Inc.

As the Protein Array Pioneer Company,
Excellence and Innovation Is Our Goal

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RayBiotech, Inc

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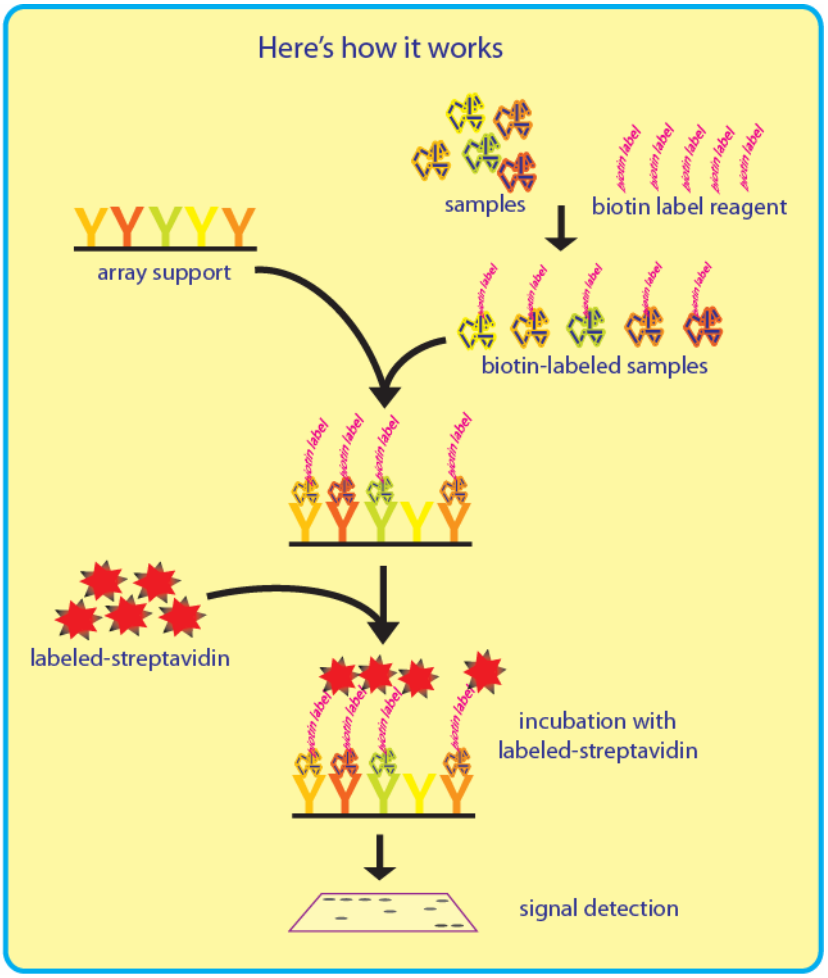
I. Introduction

Recent technological advances by Raybiotech have enabled the largest commercially available antibody array to date. With the L Series 90, researchers can now obtain a broad view of cytokine expression. The expression levels of 90 rat target proteins can be simultaneously detected, including cytokines, chemokines, adipokine, growth factors, angiogenic factors, proteases, soluble receptors, sobuble adhesion molecules and other proteins in cell culture supernates. Furthermore, an internal control is used to monitor the whole process including biotin-labeling, so this massive array will accurately reflect the available cytokines in your sample.

The first step in using the RayBio® Biotin label-based rat antibody array 1 is to biotinylate the primary amine of the proteins in cell culture supernates. The biotin-labeled sample is then added onto array membrane and incubated at room temperature. After incubation with HRP-streptavidin, the signals can be visualized by chemiluminescence.



II. Materials Provided

Upon receipt, the Box 1 should be stored at -20 °C and Box 2 should be stored at 4 °C. Please use within 6 months from the date of shipment. After initial use, the Blocking Buffer, Stop solution, HRP-Conjugated Streptavidin, Detection Buffer C and D should be stored at 4 °C to avoid repeated freeze-thaw cycles. The Array Membrane and Internal Control should be kept at -20 °C.

Box-1 (store at -20 °C):

- Labeling Reagent (Item B, 1 tube for 2 array membranes , and 2 for 4 array membranes)
- Internal control (Item C, 1 tube for 2 array membranes , and 2 for 4 array membranes)
- Stop Solution (Item D, 50 µl)
- RayBio® Biotin label-based Rat Antibody Array 1 (Item E, 2 membrane for Cat#: AAR-BLM-1-2**,** and 4 for Cat#: AAR-BLM-1-4)
- Blocking Buffer (Item F, 30 ml for each bottle, 1 bottles for 2 array membranes , and 2 for 4 array membranes)
- 500X HRP-Conjugated Streptavidin Concentrate (Item I, 100 µl)
- Detection Buffer C (Item K, 5 ml for 2 membranes, and 10 ml for 4 membranes)
- Detection Buffer D (Item L, 5 ml for 2 membranes, and 10 ml for 4 membranes)
- Plastic sheet

Box 2 (store at 4 °C):

- Dialysis tube and Floating Rack (Item A, 2 tubes for 2 array membranes, and 4 for 4 array membranes, dialysis tube is from EMD product)
- 20X Wash Buffer I (Item G, 30ml)
- 20X Wash Buffer II (Item H, 30ml)
- Spin Column (Item J, 2 columns for 2 array membranes, and 4 for 4 array membranes)
- Plate (1 plate)

III. Additional Materials Required

- 1X PBS, pH=8.0
- Shaker
- 2~5 ml tube
- 50 ml conical collection tube
- Distilled water
- Kodak X-Omat™ AR film (REF 165 1454) and film processor or Chemiluminescence imaging system

IV. Overview and General Considerations

A. Handling Array Membranes

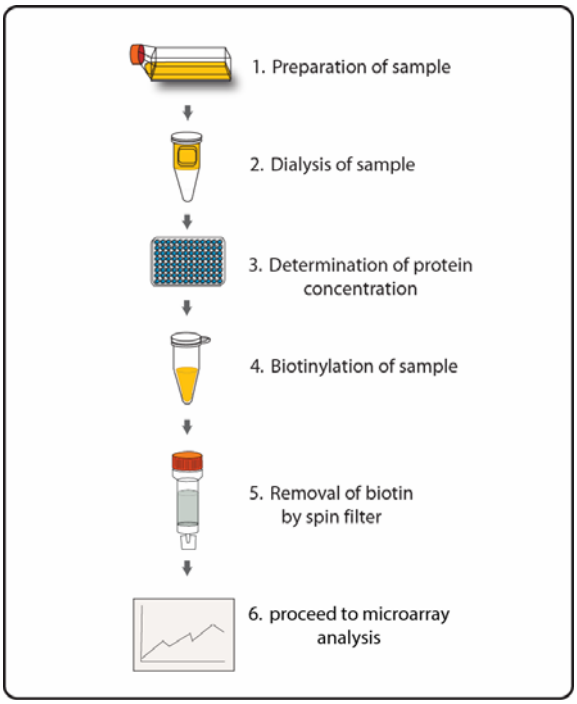
- Always use forceps to handle membranes, and grip the membranes by the edges only.
- Never allow array membranes to dry during experiments.
- Avoid touch Array membrane by hand, tips or any sharp tools.

B. Incubation

- Completely cover membranes with sample or buffer during incubation, and cover eight-well tray with lid to avoid drying.
- Avoid foaming during incubation steps.
- Perform all incubation and wash steps under gentle rotation.
- Several incubation steps such as step 3 in page 10 (sample incubation) or step 7 in page 11 (HRP-streptavidin incubation) may be done at 4 °C for overnight.

V. Protocol

Assay Diagram



A. Preparation of Samples

The cell culture supernates can be prepared in the following conventional manner:

To prepare cell culture supernates (cell conditioned media), cells are plated in 100 mm tissue culture dishes at a density of $1x10^6$ cells* per dish. The cells are then cultured with complete culture medium for 24~48 hours**. The complete culture medium is replaced with lower serum medium such as 0.2% FCS serum, and then the cells are cultured for 48 hour** again once more. The supernates are collected, centrifuged at 1,000 g, aliquoted and stored at –80 ^{0}C until use. Meanwhile, the cells are also collected and the

total protein concentration is determined. For each sample it is recommended that the concentration of the supernates and cell lysate (help nomalize different cell culture supernates) be determined using a total protein assay (BCA Protein Assay Kit, Pierce, Prod# 23227).

*Note: * The density of cells per dish used is dependent on the cell type More or less cells may be used.*

*** Culture times may vary depending on your cell lines and research.*

B. Dialysis of Sample

The cell culture supernates should be dialyzed with a Dialysis tube (Item A) before the biotin-labeling procedure. We recommend loading 2.5~3.0 ml cell culture supernates into a dialyzer and dialyzing with at least 2,000 ml 1X PBS buffer (pH = 8) at 4 ^{0}C. Change the 1X PBS buffer and dialyze again. Allow at least 3 h for each dialysis step, stir gently. The sample total volume may be changed after dialysis.

Note: Preparation of 1X PBS, pH=8.0, 1.0 g KCl, 40 g NaCl, 1.0 g KH_2PO_4, 5.75 g Na2HPO4 dissolve in 4,500 ml deionized or distilled water. Adjust pH=8.0 with 1M NaOH and adjust final volume to 5,000 ml with deionized or distilled water.

C. Biotin-labeling Sample

Avoid contamination with any solution containing amines (i.e., Tris, glycine) as well as Azide during the biotinylation process.

1. Briefly spin down Internal Control tube (Item C*)* before use.

Add 100 µl 1X PBS, pH=8.0 into the Internal Control tube, pipette up and down to dissolve the powder. Transfer 2 ml dialyzed sample into a new tube. Add 40 µl prepared Internal Control into the tube. Mix well.

2. Immediately before use, briefly spin down the Labeling Reagent tube (Item B). Add 100 µl 1X PBS into the tube, pipette up and down or vortex to dissolve the powder to prepare 1X Labeling Reagent solution.

3. Add an appropriate amount* of prepared Labeling Reagent into above tube with sample in step 2, mix well immediately. Incubate the reaction solution at room temperature for 30 min with gentle shaking. Gently tap the tube to mix the reaction solution every 5 min.

* *7.2 µl of 1X Labeling Reagent for labeling 1 mg total protein in supernates* .

Note: You need to re-calculate the total protein concentration if cell culture supernatet volume is changed after dialysis and you measure the total protein concentration before dialysis step.

4. Add 5 µl Stop Solution into the above reaction solution and then use the spin column to remove free biotin.

a). Twist off the spin column's bottom closure and loosen the cap. Place the column into a 50 ml collection tube.

b). Centrifuge column at 1,000 g for 3 minutes to remove storage solution.

Note: The resin will appear compacted after centrifugation.

c). Add 5 ml 1X PBS into column, centrifuge at 1,000 g for 3 minutes to 1X PBS. Repeat additional 2 times to wash the column.

d). Place the column in a new collection tube, slowly load the sample to the center of the compact resin bed.

e). Centrifuge the column at 1,000 g for 3 minutes to collect sample. Stored at –80 ^{0}C until testing. Discard column after use.

D. Blocking and Incubation

1. Place each membrane into the provided tray ("-" mark is on the antibody printed side).

   Note: *The printed side should be facing upward.*

2. Add 2.5 ml Blocking Buffer and incubate at room temperature for 1 hour to block membranes.

3. Decant Blocking Buffer from each container. Add 2.5 ml of sample into each array membrane, and cover with the lid. Incubate at room temperature with gentle shaking for 2 hours. Dilute sample using Blocking Buffer.

   Note: 1). We recommended using 2.5 ml of 5-fold diluted cell culture supernates which have been biotin-labeled.

Dilute sample using Blocking Buffer.

Note: 2). The amount of sample used depends on the abundance of protein. More of the sample can be used if signals are too weak. If signals are too strong, the sample can be diluted further.

Note: 3). Incubation may be done at room temperature for 2 hours. Over night at 4°C

4. Decant the samples from each container, and wash 3 times with 3 ml of 1X Wash Buffer I at room temperature with shaking. 5 min per wash. Dilute 20X Wash Buffer I with deionized or distilled water.

5. Decant the 1X Wash Buffer I from each container. Wash 3 times with 3 ml of 1X Wash Buffer II at room temperature with gentle shaking.

6. Add 2.5 ml of 500 fold diluted HRP-conjugated streptavidin (e.g. add 10 µl of HRP-conjugated streptavidin to 5 ml of Blocking Buffer) to each membrane.

Note: *Mix tube containing 500X HRP-Conjugated Streptavidin well before use since precipitation may form during storage.*

7. Incubate at room temperature with gentle shaking for 2 hours.

Note: incubation may be done at 4^0C for overnight.

8. Wash as directed in steps 4 and 5.

E. Detection

*** Do not let the membrane dry out during detection. The detection process must be completed within 40 minutes without stopping.**

1. Add 2.5 ml of Detection Buffer **C** and 2.5 ml of Detection Buffer D into a tube (for detecting 2 membranes); Mix both solutions; Drain off excess wash buffer. Place membrane protein side up ("-" mark is on the protein side top left corner) on a clean plastic plate or its cover (provided in the kit). Pipette 2.2 ml of the mixed Detection Buffer on to each membrane and incubate at room temperature with shaking for 2 minutes. Ensure that the detection mixture is evenly covering the membrane without any air bubbles.

2. Gently place the membrane with forceps, protein side up, on a piece of plastic sheet ("-" mark is on the protein side top left corner). Cover the array with another piece of plastic sheet. Gently smooth out any air bubbles. Avoid using pressure on the membrane. Work as quickly as possible.

3. The signal can be detected directly from the membrane using a chemiluminescence imaging system or by exposing the array to x-ray film (we recommend using Kodak X-Omat™ AR film) with subsequent development. Expose the membranes for 40 Seconds. Then re-expose the film according to the intensity of signals. If the signals are too strong (background too high),

reduce exposure time (eg, 5–30 seconds). If the signals are too weak, increase exposure time (eg, 5–20 min or overnight). Or re-incubate membranes overnight with 1X HRP-conjugated streptavidin, and repeat detection on the second day.

4. Save membranes at –20 °C to –80 °C for future reference.

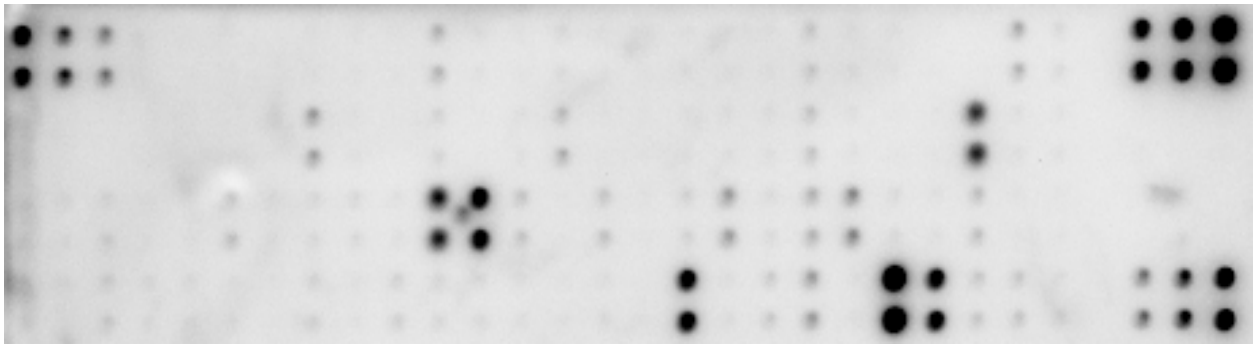
VI. Interpretation of Results:

The following figure shows the RayBio® **Biotin-label-based Array I** probed with cell culture supernates. One important parameter is the background signal. To obtain the best results, we suggest that several exposures be attempted. By comparing the signal intensities, relative expression levels of target proteins can be made. The intensities of signals can be quantified by densitometry. A biotinylated protein and internal control will produce positive control signals, which can be used to identify the orientation and help normalize the results from different arrays being compared.

Antibody affinity to its target varies significantly between antibodies. The intensity detected on the array with each antibody depends on this affinity; therefore, signal intensity comparison can be performed only within the same antibody/antigen system and not between different antibodies.

The **RayBio® Analysis Tool** is a program specifically designed for analysis of RayBio® Biotin Label-based Antibody Arrays. This tool will not only assist in compiling and organizing your data, but also reduces your calculations to a

"copy and paste." Call RayBiotech, Inc. at 770-729-2992 for ordering information.



RayBio® Biotin Label-based Rat Antibody List

1	P-1a	31	Blank	61	IL-1 beta	91	Neuropilin-2
2	P-1b	32	Blank	62	IL-1 R6/IL-1 R rp2	92	NGFR
3	P-1c	33	Blank	63	IL-2	93	Orexin A
4	Blank	34	Blank	64	IL-3	94	Osteopontin/SPP1
5	Neg	35	Blank	65	IL-4	95	PDGF-AA
6	Neg	36	Blank	66	IL-5	96	Prolactin R
7	Blank	37	Blank	67	IL-6	97	RAGE
8	Activin A	38	EG-VEGF/PK1	68	IL-10	98	RALT/MIG-6
9	ACTH	39	E-Selectin	69	IL-12/IL-23 p40	99	RELM beta
10	ADFP	40	FADD	70	IL-13	100	Resistin
11	Adiponectin/Acrp30	41	Fas Ligand/TNFSF6	71	Integrin alpha M beta 2	101	TAL1A
12	AMPK alpha 1	42	Fas/TNFRSF6	72	Insulin	102	TGF-beta1
13	B7-1/CD80	43	FGF-BP	73	IP-10	103	TGF-beta2
14	BDNF	44	Follostatin-like -1(FSL1)	74	Leptin (OB)	104	TGF-beta3
15	beta-Catenin	45	Fractalkine	75	LIX	105	Thrombospondin
16	basic-FGF	46	GFR alpha-1	76	L-Selectin/CD62L	106	TIE-2
17	beta-NGF	47	GFR alpha-2	77	MCP-1	107	TIMP-1
18	CCR4	48	GM-CSF	78	MDC	108	TIMP-2
19	CD106	49	Growth Hormone	79	MIF	109	TIMP-3
20	CINC-2 alpha/beta	50	Growth Hormone R	80	MIP-1 alpha	110	TLR4
21	CINC-3	51	Hepassocin	81	MIP-2	111	TNF-alpha
22	CNTF	52	ICAM-1/CD54	82	MIP-3 alpha	112	TRAIL
23	CNTF R alpha	53	ICK	83	MMP-13	113	TROY
24	CSK	54	Insulin Degrading Enzyme	84	MMP-2	114	Ubiquitin
25	CXCR4	55	IFN-gamma	85	MMP-8	115	VEGF
26	EGFR	56	IL-1 alpha	86	MuSK	116	VEGF-C
27	Blank	57	Blank	87	Blank	117	Blank
28	IC-3	58	Neg	88	Blank	118	P-2c
29	IC-2	59	Neg	89	Blank	119	P-2b
30	IC-1	60	Neg	90	Blank	120	P-2a

RayBio®Biotin Label-based Rat Antibody Array 1 Map

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	P-1a	P-1b	P-1c	Blank	Neg	Neg	Blank	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	Blank	IC-3	IC-2	IC-1
2	P-1a	P-1b	P-1c	Blank	Neg	Neg	Blank	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	Blank	IC-3	IC-2	IC-1
3	Blank	Blank	Blank	Blank	Blank	Blank	Blank	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	Blank	Neg	Neg	Neg
4	Blank	Blank	Blank	Blank	Blank	Blank	Blank	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	Blank	Neg	Neg	Neg
5	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	Blank	Blank	Blank	Blank
6	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	Blank	Blank	Blank	Blank
7	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	Blank	P-2c	P-2b	P-2a
8	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	Blank	P-2c	P-2b	P-2a

VII. Troubleshooting Guide

Problem	Cause	Recommendation
Weak signal or no signal	1. Taking too much time for Detection.	1. The whole Detection process must be completed in 30 min.
	2. Film developer does not work properly.	2. Fix film developer.
	3. Did not mix HRP-streptavidin well before use.	3. Mix tube containing HRP-Conjugate Streptavidin well before use since precipitates may form during storage.
	4. Sample is too dilute.	4. Increase sample concentration
	5. Other.	1.Reduce blocking concentration by diluting in 1X Wash Buffer II.
		2. Slightly increase HRP concentrations.
		3. Slightly increase biotinylate-antibody concentrations.
		4. Expose film for overnight to detect weak signal.
Uneven signal	1. Bubbles formed during incubation.	1. Remove bubbles during incubation.
	2. Membranes were not completely covered by solution.	2. Completely cover membranes with solution.
High background	1. Exposure to x-ray file is too long.	1. Decrease exposure time.
	2. Membranes were allowed to dry out during experiment.	2. Completely cover membranes with solution during experiment.
	3. Sample is too concentrated.	3. Use more diluted sample.

VIII. Reference List

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